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SPECIAL REPORT TO DEPARTMENT OF THE NAVY, BUREAU OF YARDS AND DOCKS. SPORICIDAL ACTION OF FREE AVAILABLE CHLORINE

A. Richard Brazis, et al

Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio

April 1957

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BUREAU OF YARDS AND DOCKS

SPORICIDAL ACTION OF FREE AVAILABLE CHLORINE

by

A. Richard Braxis, James E. Leslie
Paul W. Kabler, and Richard L. Woodward

Project NY 300 010 Sub-project 6

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U. S. Department of Health, Education, and Welfare
Public Health Service
Robert A. Taft Sanitary Engineering Center

April, 1957

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SPECIAL REPORT

TO

DEPARTMENT OF THE NAVY
BUREAU OF YARDS AND DOCKS

I. SPORICIDAL ACTION OF FREE AVAILABLE CHLORINE

Effect of Free Available Chlorine Upon *Bacillus globigii* and *Bacillus anthracis* Spores at Varying pH, Temperature, and Holding Periods

by

A. Richard Brazis, James E. Leslie - m. ent.
Paul W. Kabler, and Richard L. Woodward - et. a.

Project NY 300 010 Sub-project 6

1. Water Purif - Chlorination

U. S. Department of Health, Education, and Welfare
Public Health Service
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April, 1957

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ABSTRACT

The effect of free available chlorine upon test waters containing spores of Bacillus globigii and Bacillus anthracis was determined. Using contact periods of two hours and forty-eight hours, test waters containing free available chlorine and spores were evaluated at hydrogen-ion concentrations of 6.2, 7.2, 8.6 and 10.3-10.5 and storage temperatures of 4° and 22°C. The amount of free available chlorine required to achieve a 99.99% kill under the above mentioned conditions was determined.

Special procedures were developed for cultivation and standardization of spore suspensions, germination and sporulation media, and high quality synthetic buffered test waters.

SUMMARY

1. Spore suspensions of Bacillus globigii and Bacillus anthracis were prepared by utilization of highly specified sporulation media.
2. Media were developed for maximum germination of test spores following exposure to the action of free available chlorine.
3. Vegetative cells and granular material were removed from the spore suspensions by autolytic methods.
4. Standardization and purification of spore suspensions were achieved by centrifugal washings and filtration.
5. Hydrogen-ion concentrations, in the pH range of 10.3 to 11.2 were shown not to contribute to the death of the test spore suspensions.
6. Bacterial spores of B. globigii and B. anthracis were exposed to sporicidal free available chlorine during contact periods of two and forty-eight hours, at hydrogen-ion concentrations of 6.2, 7.2, 8.6, and 10.3-10.5, and at temperatures of 4° and 22°C., with results as follows:
   a. As the pH level increased from 6.2 to 10.5, at 22°C., the amount of free available chlorine required to produce a 99.99% kill of B. globigii spores in 90 minutes, increased from 1.9 to more than 454 p.p.m.
   b. Lowering the temperature from 22° to 4°C., resulted in increasing the concentration of free available chlorine required to produce a 99.99% kill. At 22°C., B. globigii spores required 1.9 and 2.3 p.p.m. free available chlorine at pH levels of 6.2 and 7.2, respectively, for 90 minutes, and at 4°C., they required 6.8 and 9.0 p.p.m. free available chlorine, respectively.

c. Increasing the length of the contact period, from two to forty-eight hours, resulted in decreasing the concentration of free available chlorine required to produce a 99.99% kill. At pH 8.6 and 22°C., B. globigii spores required 25 p.p.m. free available chlorine for 90 minutes and 1.2 p.p.m. free available chlorine for 30 hours, to reach a 99.99% kill.

d. Free available chlorine was much more sporicidal for B. anthracis spores than for B. globigii spores, except at pH 10.3. At pH 7.2 and 22°C., 0.33 p.p.m. free available chlorine destroyed 99.99% of B. anthracis and B. globigii spores in 6.5 and 14 hours, respectively.

e. When the amount of free available chlorine used in these observations was evaluated in terms of hypochlorous acid, it was found that approximately the same concentration of HOCl was required at all pH levels to achieve a 99.99% kill. Maintenance of 0.3 and 0.7 p.p.m. total sporicidal residual hypochlorous acid during a contact period of 48 hours at 22° and 4°C., respectively, resulted in satisfactory treatment of stored waters at pH reactions less than 9.0. Concentrations of 2.0 and 7.0 p.p.m. total sporicidal residual hypochlorous acid at pH reactions less than 9.0, in waters stored for two hours at 22° and 4°C., achieved the same results.

f. Mouse LD_{50} titrations were used to demonstrate the degree of pathogenicity present in the strain of B. anthracis studied. Chlorination did not affect the pathogenicity of surviving organisms.

INTRODUCTION

Spores of microorganisms belonging to the Bacillus group are more resistant to the chemical disinfectants than other microorganisms. A representative, saprophytic indicator bacillus has been used in this study. Its resistance, in the spore form, has been demonstrated to be greater than that of the pathogenic members of this group.

Studies involving the effect of "free" chlorine upon the spores of a saprophytic aerobe, B. globigii, and a pathogenic aerobe, Bacillus anthracis, under varying conditions of hydrogen-ion concentration, temperature, and length of exposure, were initiated at the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio in order to provide necessary information for utilization by water treatment plants in times of disaster. Chlorination adequate to deal with these forms will also kill other microorganisms.

REVIEW OF LITERATURE

Quality of Water Tested

Early investigators used synthetic test waters in evaluating the germicidal action of chlorine and chlorine bearing disinfectants but neglected two important variables: pH and the distinction between free and combined chlorine. Tonney, Greer, and Danforth[1], and Tonney, Greer and Liebig[2] measured the minimal chlorine death values of vegetative cells and spores of a number of genera of microorganisms. There was no attempt to control the hydrogen-ion concentration of the test waters. Tap water was used by Heathman, Pierce and Kabler[3] in order to study the resistance

of coliforms and Eberthella typhosa to chlorine and chloramines. Since the work of these investigators involved questionable chlorine-demand-free water, their results cannot be accepted as indicative of the effect of chlorine or chloramine alone.

The test water used by Butterfield, Wattie, Megregian and Chambers[4] was essentially the same as was used by Megregian[5].

Traces of inorganic or organic material present on the internal sides of flasks used during test will interfere significantly with maintained chlorine residuals. Butterfield et al[4] showed that even the condensate from an autoclave will result in a chlorine demand of 0.01 to 0.05 p.p.m. when chlorine demand free test water containing a known chlorine residual is added.

Methodology in Determining Germicidal Kill

Many methods have been suggested and used for determining the germicidal activity of available chlorine containing compounds. Methods such as the Rideal Walker, Hygienic Laboratory, and Food and Drug Administration have been suggested and used for this purpose. These methods are quite limited, however, in their applicability towards free available chlorine. Johns[6] developed a method that simulated the actual field conditions under which the germicidal activity of chlorine versus the predominating flora of microorganisms could be measured and is known as the Johns' Glass Slide Method. A laboratory procedure developed by Weber and Black[7], evaluated the germicidal activity performed in sanitizing food utensils. Chambers[8] modified the Butterfield et al.[4] procedure further by the following important changes: (a) larger quantities of water were used to provide

material for chemical control tests; (b) more than one test temperature was used; and (c) suspension of E. coli cells was filtered through sterile filter paper to remove clumps, eliminating most of the "freak jumps".

Cultivation and Standardization of Spore Suspensions

A number of different cultivation and standardization procedures for spores can be used to obtain stock suspensions of B. globigii and B. anthracis. Germicidal studies conducted in 1930 by Tilley and Chapin[9] used spores of B. anthracis as the test suspension. The surface growth from two to three week old agar cultures was suspended in sterile normal saline solution as a stock suspension and stored at 4°C. Roberts and Baldwin[10] have shown that certain hydrophilic colloids, such as agar and hydrophobic colloids, such as iron, silica and charcoal, will appreciably increase sporulation by Bacillus subtilis. Sporulation on infusion agar slopes by B. subtilis and B. cohaerens was obtained by Curran and Evans[11].

In studies with B. metiens, Rudolph and Levine[12] washed the growth from 20 day old nutrient agar cultures and filtered the suspension through sterile filter paper to remove clumps. Heat shock, at 80°C. for ten minutes, killed the vegetative cells present. The suspensions were washed and then placed in Butterfield's dilution "C" water. The suspensions were stored at 4°C. until used. Complete sporulation by B. anthracis on agar slants incubated for 2-3 weeks was observed by Jones[13]. Evans and Curran[14], and Curran and Evans[15] used sublethal preincubation heating at 85°C. for 8 to 10 minutes, as a means to accelerate the germination of spores of B. megatherium, B. cereus, B. subtilis, B. cohaerens, and B. fusiformis, on glucose agar plates.

Brewer et al.[16, 17] studied the nutritional requirements of B. anthracis. Their work showed that a basal medium containing a source of calcium, iron, magnesium and manganese trace minerals, plus pepticase, peptone, cerelose and mono- and di-potassium phosphate proved to be very desirable. Hills[18] found that the slow germination of spores of B. anthracis in the basal medium was accelerated significantly by addition of yeast extract equivalent to 0.01% fresh yeast.

According to Stewart, Taylor and Halvorson[19], free spores of aerobic spore-forming bacilli contain a highly active enzyme similar to the alanine racemase found in Streptococcus faecalis. This enzyme is responsible for regulating the number of spores that germinate at any one time. Using heat shock to activate spore germination, it was found that after heating at 65°C. for 15 minutes, no appreciable enzyme activity was lost. After 120 minutes heating at 80°C., the activity dropped to 97 percent of its original value. Using suitable additions of soluble iron or manganese salts to fluid organic media, Curran and Evans[20] state that the total spore yields of B. subtilis, were greatly increased. In later work completed by Church, Halvorson and Halvorson[21], B. globigii spores were obtained by growth on brain heart infusion broth and a special "G" medium containing soluble salts of ammonia, manganese, magnesium, zinc, copper, calcium, and iron. The autolysis of vegetative cells from the spores was hastened by incubating the suspension resuspended in sterile distilled water at temperatures above the growth temperature.

Lysogenic Autolysis

Lysozyme has been used by different investigators as a means of bringing about increased autolysis of vegetative cells. In studies with suspensions

of B. megatherium, Welshimer and Robinow[22] treated the cells with 2% formaldehyde, washed and resuspended in M/15 phosphate water, then added a solution of lysozyme to give a final concentration of 4 micrograms/ml. of suspension. After incubation at 34°C., lysozyme was inactivated by adding 2 drops of formalin. Suspensions showed visible reduction of turbidity in 10 minutes and were cleared in 30 minutes.

Thirteen strains of B. anthracis were tested for sensitivity to lysozyme by Gladstone and Johnston [23]. No strain was found to be susceptible when grown under ordinary laboratory conditions in air. Five strains were highly susceptible when cultured in 10-20 percent CO_2 in a medium containing 0.05-0.1 M HCO_3^-.

Effect of Chlorine on Vegetative Cells

In early investigations the germicidal activity of chlorine was measured with no attempt being made to stabilize the hydrogen-ion concentration of test solutions. Due to a lack of knowledge concerning marked differences between free and combined chlorine, much of the work accomplished up to 1941 showed only that chlorine destroyed vegetative cells and spores of bacteria. The effect of chlorine upon vegetative cells and spores of bacteria, viruses and cysts, as reported by various investigators, can be seen in Table I. As indicated in Table I, exposure time refers to the time required to inactivate 99% or more of the saprophytes and pathogens studied. Investigations by Darnall[24] indicated that most organisms were inactivated by the action of chlorine, except spores of B. subtilis which resisted chlorine action even in large amounts and after long contact. Tonney, et al.[1], determined the minimal chlorine death points of vegetative cells and reported that of all the organisms studied, E. coli was the most resistant. Working with

Clorina-Heyden (Chloramine-T) and hypochlorites, Heine[25] experimented with their effects upon B. anthracis, B. subtilis, Staphylococcus aureus and E. coli. Pasteurella tularensis was rendered inactive through action with chlorine by Foote, Jellison, Steinhaus and Kohls[26]. The effect of halazone and calcium hypochlorite on Leptospira icterohaemorrhagiae was observed by Chang, Buckingham and Taylor[27]. Calcium hypochlorite killed all the leptospira in one and three minutes when the chlorine residuals were 0.5 and 0.3 p.p.m. respectively.

Butterfield et al.[4] studied the relative survival of E. coli, A. aerogenes, Pseudomonas pyocanea, E. typhosa and S. dysenteriae when exposed to free chlorine waters at four different hydrogen-ion concentrations, see Table I.

Clarke and Kabler[28] demonstrated that a purified suspension of Coxsackie virus in water can be inactivated by chlorine. Substantially more free chlorine was required to achieve comparable kills of Coxsackie virus than is required to kill E. coli cells under parallel conditions of temperature and hydrogen-ion concentration.

Effect of Chlorine on Bacterial Spores

Early studies involving the effect of chlorine on bacterial spores followed somewhat the same pattern used in studying vegetative cells. Hydrogen-ion concentration and free chlorine were not evaluated as important variables in germicidal kills. Tonney, Greer and Liebig[2] reported that the chlorine death points of bacterial spores ranged from 2.5 p.p.m. for Clostridium welchii to 280 p.p.m. for B. vulgatus. Studies conducted by Tilley and Chapin[9] on anthrax spores, showed that nitrogen trichloride, chlorine in neutral solutions and chlorine plus N/50 HCl were effective in 15 minutes with available

Table I

Germicidal Effect of Chlorine Upon Microbiological Saprophytes and Pathogens

Organism	Investigator(s)	pH	Temp.	Exposure Time(min)	Chlorine (ppm) Free	Total
Spores:						
Bacillus metiens	Charlton et al(34)	8.3	25°C	5		20
Bacillus metiens	Weber(30)	5.0-7.0	"	2.1-3.0		22-24
		8.0	"	7.6		22-24
		9.0	"	58		22-24
		10.0	"	570		22-24
Bacillus anthracis	Fair et al(31)	5.0	"	10	15	
		5.0	"	15	8.2	
		5.0	"	20	5.3	
		5.0	"	30	3.0	
		5.0	"	40	1.9	
		5.0	"	50	1.4	
		5.0	"	60	1.0	
Clostridium welchii	Tonney et al(2)	-	-	15-30 sec.		160.00
Cysts:						
Endamoeba histolytica	Fair et al(31)	5.0	23°C	30	1.2	
		6.0	"	"	1.2	
		7.0	"	"	1.6	
		8.0	"	"	5.5	
		9.0	"	"	40.0	
		10.0	"	"	230.0	
Endamoeba histolytica	Metzler et al(35)	-	-	360	0.35	
				60	1.8	
Vegetative Cells:						
Pasteurella tularensis	Foote et al(26)	-	-	30		0.2
Leptospira icterohaemorrhagiae	Chang et al(27)	5.0	-	1	0.5	
		5.0	-	3	0.3	
		8.0	-	1	6.0	
		8.0	-	3	3.0	
Escherichia coli	Butterfield et al(4)	7.0	20-25	1	0.055	
		8.5	"	10	0.1-0.29	
		9.8	"	20	0.1-0.29	
Eberthella typhosa	Butterfield et al(4)	8.5	"	3	0.1-0.29	
		9.8	"	5	0.1-0.29	
Viruses:						
Coxsackie	Clarke et al(28)	7.0	3-6°C	9.7	0.62	
		7.0	27-29	3.8	0.18	
		9.0	3-6°C	24.0	2.0	
		9.0	27-29	9.6	0.14	

chlorine concentrations of 10 p.p.m. or less. In a more recent study conducted by Fair, Morris and Chang(31), the effects of titrable free residual chlorine upon B. anthracis spores has been determined, as can be seen in Table I.

Hydrogen-ion concentration seemed to be the greatest single factor affecting the germicidal activity of chlorine against B. metiens spores, according to Charlton and Levine(34). The killing times of various concentrations of calcium hypo-chlorite were 64 minutes, 70 minutes and 5 minutes for solutions of 1000 p.p.m. at pH 11.3, 100 p.p.m. at pH 10.4, and 20 p.p.m. at pH 8.3, respectively. Charlton and Levine(37), in a later paper, acknowledged that the germicidal kill at a hydrogen-ion concentration of pH 7.0 as compared to the kill at pH 11.0 was approximately proportional to the calculated amount of hypochlorous acid present. This appeared to explain the fact that available chlorine solutions of 1000 and 100 p.p.m. made from commercial hypochlorite solutions were not appreciably different in their killing times. The killing action of hypochlorite upon B. metiens spores has been considered by Rudolph and Levine(29) to proceed in two phases: (1) The penetration of the active principle into the cell, which is more rapid with greater concentrations of undissociated hypochlorous acid, and (2) The chemical union of this principle with the protoplasm, which is directly responsible for the death of the cell. Based on the amount of hypochlorous acid present, rather than p.p.m. of free chlorine, Moore(32) states that for spores of B. anthracis and B. metiens at 25°C., 30 minutes contact, the HOCl residuals required for 100 percent kill are 3 p.p.m. and 2 p.p.m. respectively.

The effect of temperature as well as hydrogen-ion concentration upon germicidal efficiency in destroying B. metiens spores was reported by Rudolph and Levine[29]. Using a chlorine concentration of 25 p.p.m., they reported that the killing time was reduced from 50-75 percent during a rise of 10°C., from their initial storage temperature. The relationship between pH and storage time was also studied by Weber[30], see Table I.

In view of the limited knowledge afforded by previous investigations, these studies were designed to determine the effect of "free" available chlorine upon spore-forming bacteria under the following conditions: (1) Hydrogen-ion concentrations of 6.2, 7.2, 8.6 and 10.5; (2) Storage temperatures of 22° and 4°C., and (3) Storage exposure periods of 2 and 48 hours.

Preliminary studies were initiated to study many of the problems which had not been clearly defined in previous studies by other investigators. These studies included: (1) Checking distilled waters to determine their suitability for use in germicide testing; (2) Presence of endogenous respiration as a reason for "skips" in germicidal kill of spores; (3) Maintaining desirable buffer capacity of buffered synthetic test water after addition of chlorine and test spores over exposure periods of 2 to 48 hours and at temperatures of 4° and 22°C.; (4) Utilization of (specific) sporulation and germination media; (5) Employment of characteristic sporulation, filtration and centrifugation methodology to insure clean viable spore suspensions; and (6) Demonstration of non-alkaline inactivation of test spore suspensions at high pH concentrations.

MATERIALS AND METHODOLOGY

Preparation of Materials

Glassware:

All glassware was cleaned by the following procedure:

1. Fill with strong, fresh cleaning solution (sulfuric-bichromate mixture).
2. Fill and drain at least 10 times with distilled water.
3. Drain and sterilize in hot air oven. When continued sterility is desired, cap open glassware with paper and aluminum foil prior to hot air sterilization.

Buffers:

Preliminary studies showed that the buffer systems used were not toxic to the test spores and retained a constant pH reaction during long periods of holding. The buffer systems selected were taken from "Standard Methods for the Examination of Water and Sewage, 9th edition"(33).

Buffer solutions (0.2 M and 1.0 M) and salt combinations were evaluated, and buffer salts were used finally as an added convenience in preparing buffered test waters. Buffer solutions were less stable than the dry salts. The buffer capacity of the buffers used was quite stable at pH ranges of 6.0 to 8.6. Use of boric acid, potassium chloride salts, and a sizable amount of 1.0 M sodium hydroxide resulted in a stable, high buffer capacity, test water. The test water was prepared in 4-liter aliquots as shown in the following table:

pH	KH_2PO_4 (grams)	H_3BO_3 (grams)	KCl (grams)	1 M NaOH (ml.)	0.2 M NaOH (ml.)
6.2	27.2320			34.4	6.7
7.2	27.2320			120.0	7.1
8.6		12.404	14.912	48.0	8.0
10.5		12.404	14.912	194.0	

All hydrogen-ion determinations were made electrometrically using a Beckman Model H-2 pH meter. All readings were made in triplicate at each sampling interval.

The toxicity of boric acid and potassium chloride buffered test waters for B. globigii spores at pH ranges of 10.3 to 11.2 were determined during contact exposure period of 48 hours at 22°C. B. anthracis spores were exposed to pH ranges of 10.3 to 10.6 under the same conditions. Standard plate counts made on both test spore suspensions prior to and at the end of the exposure period showed no reduction in the number of viable spores present.

Neutralizer:

As a means of demonstrating non-toxicity of the neutralizer, the following procedure was used:

1. Prepare neutralizer blanks containing 5 ml. of sodium thiosulphate in amounts of 2 and 4 grams per liter. Adjust the reaction to pH 7.0.
2. Prepare 5 ml. test water blanks (sterile) at pH reactions of 6.2, 7.2, 8.6 and 10.5 and temperature of 22°C.
3. Add spore suspension of B. globigii to each test water blank. Should be 1,000 spores present per ml. of test water. Mix.

4. Using sampling intervals of 0 minutes, 1, 2, 4, and 6 hours, place 5 ml. aliquots of test water mixture into neutralizer tubes. Mix.
5. Plate from neutralizer tubes, using following sampling frequency: 0, 4, 8, 16, and 20 minutes.
6. Also plate out duplicate dilutions of initial numbers organisms in original test water blanks.
7. Incubate plates at 37°C., for 48 hours.

In demonstrating the effectiveness of the neutralizer, use the same procedure but add enough chlorine to the test water blanks to provide 20 p.p.m. free available chlorine. Use controls on test waters not containing chlorine.

According to the results received, sodium thiosulfate exhibited no apparent toxicity to the test spore suspensions. A safety factor of 200% was used in some checks, so that the neutralizer would, in effect, neutralize twice the amount of free chlorine used in any one evaluation.

Chlorine Determinations:

The determination of free and combined chlorine was made with a modification of the standard orthotolidine test(38): Fifty ml. of the test water were added to 3 ml. of orthotolidine in a 125 ml. Erlenmeyer flask. This procedure was carried out in duplicate. One of the Erlenmeyer flasks was stored in the dark for 20 minutes. The remaining flask, after standing 3 seconds, received 3 ml. of sodium arsenite solution, added quickly. The former flask also received 3 ml. of the arsenite solution at the end of the 20 minute storage period. The yellow color in the flasks was measured at 435 μ in a Beckman Model B. spectrophotometer, calibrated previously with

chlorine solutions of known concentrations. A Wallace and Tiernan Amperometric Titrator was used to standardize the spectrophotometer. Triplicate readings of chlorine concentrations, both free and combined, were made using the modified orthotolidine and orthotolidine-arsenite methods. In tests, involving chlorine concentrations higher than 20.0 p.p.m., the starch-iodometric method(33) was applied. In all the experiments shown, free chlorine values were not appreciably different from the total chlorine values. It was not possible to measure both free and combined chlorine content by the iodometric method; only free chlorine concentrations have been reported.

Spectrophotometric determinations of test water aliquots were made at two temperatures, 22° and 4°C. Samples containing high free chlorine residuals were diluted with demand-free dilution water. At 22°C., there were no technical difficulties. At 4°C., it was necessary to cool the diluent down to 4°C., add the test water and allow 10 to 15 minutes before representative samples could be taken and orthotolidine added.

Maintenance of constant free available chlorine residual values throughout the 48 hour storage period, was not possible at 22° or 4°C. Free chlorine residuals, after satisfying the initial demand of the spore suspensions, remained faily constant from 2-4 hours. Low chlorine residuals in the range of 0.2 to 1 p.p.m. became significantly reduced during the remainder of the storage period. Chlorine residuals which were initially higher also showed measurable reduction throughout the storage periods.

Stock Chlorine Solutions:

Stock chlorine solutions were prepared by passing chlorine gas through chlorine-demand-free distilled water followed by storage at 4°C.

Preparation of Chlorine-Demand-Free Water:

Chlorine-demand-free test water was prepared similar to that of Megregian(5), with one exception, dechlorination being attained by use of direct sunlight or sun lamps.

Tests for Oxidizing Agents:

Two tests were selected for evaluating the distilled water. Test #1(39) demonstrated the presence or absence of an oxidizing agent. Test #2 involved the utilization of a Barnstead Purity Meter, Model PM-2B, for measuring the conductivity of the water in terms p.p.m. as NaCl. Desirable distilled water contained no higher than 1 p.p.m. NaCl conductivity.

Preparation of Spore Suspensions:

Development of Media:

The strain of B. anthracis used was obtained from Ohio State University, #811. It was isolated from a sow on February 23, 1952. The strain of B. globigii was obtained from Ohio State University #102 with the designated nomenclature: B. subtilis variety niger. Both names represent the same entity.

Initial studies with spore-forming microorganisms involved the testing of four media which would induce rapid germination during incubation at 37°C. Three of these media were used with B. globigii spores, and two were used with B. anthracis spores. The media included: milk protein hydrolysate agar, milk protein hydrolysate agar plus 0.5% yeast extract, nutrient agar plus 0.5% yeast extract and 0.3% sodium citrate, and tryptose agar plus 0.2% liver extract. Spore suspensions were added to the above named media and plate counts were made at the end of 24, 48, and 72 hours to ascertain which medium provided the best facility of germination. As

seen in Table II, nutrient agar plus 0.5% yeast extract and 0.3% sodium citrate appeared to be best suitable for B. globigii spores. Tryptose agar plus 0.2% liver extract proved to be best for B. anthracis. Spores of two other organisms, B. subtilis and B. cereus, were used primarily for comparative growth studies with the test media under observation. The above named media were used in all studies involving the germicidal action of free available chlorine.

Determination of media which would bring about maximum sporulation of the test organisms under suitable conditions was made. Ten highly nutritious media[21,16,17], were evaluated as regards the development of maximum numbers of mature spores. Growth was maintained primarily on nutrient agar slants or glucose yeast agar slants prior to inoculation onto the different test media. Agar was used in the amount of 3% during sporulation to provide a solid surface which would not crumble during the washing phases. Table III shows that medium #9, which had been adjusted to pH 6.8 with 1.0 M sodium hydroxide, provided the greatest number of mature viable spores.

Vegetative Cell Autolysis:

The presence of vegetative cells in spore suspensions might lead to endogenous respiration, resulting in germination of spores during a free chlorine evaluation. Germination of the spore suspensions in different test waters under conditions of the investigation could result in false conclusions at to the sporicidal efficiency of free chlorine. Three methods of increasing vegetative cell autolysis were evaluated: heat shock, lysozyme action, and incubation at higher than normal growth temperatures.

Table II

Average Standard Plate Counts of Freshly Prepared Spores and Vegetative Cells (millions per ml.) Grown on Sporulation Media

Organism	S M*	Nutrient Yeast Agar	MPH Yeast Agar
Bacillus subtilis	#1	20	11
" "	#2	92	94
" "	#4	55	61
" "	#5	110	100
Bacillus cereus	#1	300	270
" "	#2	110	82
" "	#7	0.47	0.44
" "	#8	0.61	0.56
" "	#9	7.4	<3
Bacillus globigii	#2	19	23
" "	#4	13	10
" "	#5	8	7
" "	#7	1	1
" "	#8	32	25
" "	#9	1,200	830

*S M = Sporulation Media

#1. Brain Heart Infusion Broth
#2. Special "G" Bacillus Nutrient Broth
#4. 0.1% Casamino Acid, 0.25% Glucose and 0.5% Yeast Extract Agar
#5. 0.1% Casamino Acid, 0.5% Yeast Extract Agar
#7. 0.25% Glucose, 0.5% Yeast Extract Agar
#8. 0.25% Glucose Agar
#9. 0.1% Casamino Acid, 0.25% Glucose, 0.5% Yeast Extract, 0.01% Manganese Sulfate, 0.0001% Ferrous Sulfate Agar.

Table III

Standard Plate Counts/ml. of Spores and Vegetative Cells Grown on Nutrient Yeast Agar or Milk Protein Hydrolysate plus Yeast Extract (all figures X 10^5)

Organism	Media*								
	#1	#2	#3	#4	#5	#6	#7	#8	#9
Bacillus subtilis	200	940	180	610	1,100	<30	<3	<3	<30
Bacillus cereus	3,000	1,100	170	1,600	1,500	540	5	6	70
Bacillus globigii	<30	230	<30	<130	80	<30	10	320	12,000

* #1. Brain Heart Infusion Broth
#2. Special "G" Bacillus Nutrient Broth
#3. 0.1% Casamino Acid Agar
#4. 0.1% Casamino Acid, 0.25% Glucose and 0.5% Yeast Extract Agar
#5. 0.1% Casamino Acid, 0.5% Yeast Extract Agar
#6. 0.1% Casamino Acid, 0.25 Glucose Agar
#7. 0.25% Glucose, 0.5% Yeast Extract Agar
#8. 0.25% Glucose Agar
#9. 0.25% Glucose, 0.1% Casamino Acid, 0.5% Yeast Extract, 0.01% Manganese Sulfate, 0.0001% Ferrous Sulfate Agar.

Vegetative cell autolysis obtained by a combination of heat shock plus incubation at 45°C., for 24 hours, proved to be the most efficient method for breaking down vegetative cells. As seen in the results obtained during the purification of spore suspensions, about 99.9% of the vegetative cells were effectively removed during the washing process which followed cellular autolysis. Lysozyme did not prove to be effective in lysing the vegetative cells of B. globigii.

Purification and Standardization:

The procedure used was based on harvesting spores from two sources of sporulation media: (a) one liter agar slants and (b) 20 ml. agar slants. When using one-liter agar slants, it was necessary to use large quantities of sterile double distilled wash water. One-liter agar slants were used where large numbers of spores were required. One large agar slant eliminated the necessity of using fifty 20 ml. agar slants. The type of container used for large recovery of spores was the Corning Diphtheria Toxin bottle(41). For collecting smaller amounts of spores, or for inoculating the larger type bottles, Corning milk dilution bottles were used. The procedure used followed this outline:

1. Streak test organisms, either B. globigii or B. anthracis on glucose yeast agar tube slants (6 ml./tube)and incubate at 37°C. for 48 hours.
2. Remove from incubator and wash organisms free from tube slant with 5 ml. of sterile double distilled water.
3. Place washed organisms (5 ml.) from previous step, on milk dilution bottle slants (20 ml.). Sporulation agar should be

used here and in any later steps. Excess suspension rinse should be drained off prior to incubation.

4. Where incubation in the small milk dilution bottles was the last step in sporulation prior to purification of the suspension, bottles were incubated in inverted position at 37°C. for 5 days.

   Where toxin type bottles were used for bulk culture, the milk dilution bottles were incubated at 37°C. for 48 hours.

   a. Following incubation period, rinse dilution bottle slants with 20 ml. of sterile double distilled water. Transfer this rinse suspension to the toxin type bottle slant and completely expose all surfaces. Drain off excess rinse suspension. Use complete safety in handling anthrax organisms, and autoclave all rinse waters and glassware used during the cultivation of B. anthracis spores. Incubate bottle slants at 37°C. for 5 days.

5. After incubation, wash milk dilution bottle slants (4) and toxin type bottle slants (4a) with 10 ml. and 500 ml. of sterile double distilled water, respectively. (Using the toxin type bottle, rinse once using 300 ml. and then 200 ml. the second time.)

   a. Make slides and check spore-vegetative cell ratio, type of debris (granular or cellular), and degree of clumping. For spore staining purposes use either Bartholomew and Mittwer's "Cold" method(40) or Snyder's modification of the Dorner method(40).

6. Transfer spore suspensions into sterile screw cap culture tubes (25 x 200 mm.) containing glass beads (5 mm. diameter). Use rotary shaker or perform manually for 20 minutes in order to break up cell mats and clumps.

7. Filter through sterile Reeve Angel agar-agar filter paper[36] or Whatman No. 2 filter paper in a safety hood, using sterile funnels, rubber policemen and forceps. Use sterile 50 ml. centrifuge tubes as the collecting tubes.

   a. When filtering 10 ml. aliquots from the milk dilution bottle slants, combine the contents from 3 bottles (30 ml.) into one centrifuge tube.

8. After filtration, make slides (5a) and compare results. Reincubate spore suspensions, in centrifuge tubes, at 45°C. for 24 hours. This hastened vegetative cell autolysis.

9. After incubation, make slides (5a) and compare results. Centrifuge suspensions at 1800 r.p.m. for 30 minutes at 5°C. (Use refrigerated centrifuge.) Draw off supernatant and resuspend suspension in 30 ml. of sterile double distilled water. Make slides (5a) and compare results.

10. Heat Shock:

    a. B. globigii suspensions were heat shocked by placing in a constant temperature water bath at room temperature, raising the temperature to 82°C. and holding for 10 minutes followed by cooling rapidly to 26°C.

    b. Suspensions of B. anthracis were heat shocked at a lower temperature, 65°C. for 5 minutes, followed by rapid cooling.

11. Centrifuge spore suspension at 5°C. for 20 minutes, five times at 1850 r.p.m. Resuspend spores after each centrifugation in 30 ml. of sterile double distilled water. Make slides (5a) and compare results.
12. Plate out spore suspensions, using dilutions up to 10^{-7}, on yeast (0.5%) nutrient agar in the case of B. globigii and liver extract (0.2%) tryptose agar when plating B. anthracis. Use sterile phosphate buffered (pH 7.0) dilution water. Incubate plates at 37°C. for 24-72 hours. Use 1.5% agar as a suitable overlay medium on all plates prepared.
13. Store spore suspensions in sterile screw cap culture tubes at 4°C. until ready for use. Repeat heat shock procedure for specific bacillus species every three months to insure pure spore suspensions.

High titres of spore suspensions were successfully cleaned of cellular and nutritive debris when the purification procedure was used. Table IV shows the effect of each stage of the procedure in removing nutritive material. Following purification of spore suspensions, stock suspensions were prepared containing 30,000,000 spores per ml. and placed in storage at 4°C., until used.

Mouse I.P. LD_{50} Titrations:

The pathogenicity of anthrax spore suspensions was determined in two studies, using white Swiss mice, males only, weighing 18-20 grams.

Previously prepared dilutions of anthrax spores, contained in 60 ml. vaccine vials, were used in these experiments. Intra-peritoneal injections of 0.2 ml. were given to mice in lots of 12 and 8. The mice were placed in stainless steel cages, 4 to a cage, and observed during a period of zero to ten days.

Table IV

Effect of Purification Process Upon Preparation of Spore Suspensions

Stage	Suspension	Ratio Spore/Veg. Cells	Debris		Degree of Clumping	DMC* Spore Count/ml.
			Amount	Type		
Washed cultures prior to shaking with glass beads	a.	2:1	much	granular	little	150 X 10^6
	b.	9:1	much	granular	little	340 X 10^6
	c.	1:150	much	cellular & granular	moderate	220 X 10^6
Shaken with beads prior to 1st filtration	a.	1:2	moderate	granular	little	110 X 10^6
	b.	5:1	much	granular	little	280 X 10^6
	c.	1:300	much	cellular & granular	moderate	150 X 10^6
Following filtration through filter paper	a.	2:1	much	fine granular	little	84 X 10^6
	b.	1:75	much	granular	little	190 X 10^6
	c,	20:1	much	fine granular	little	170 X 10^6
	A.**	2:1	much	fine granular	little	190 X 10^6
Following incubation at 45°C. for 24 hrs.	A.	20:1	moderate	fine granular	none seen	190 X 10^6
Following centrifugation & washing prior to heat shock	A.	6:1	little	granular	little	230 X 10^6
	Aa.***	15:1	very little	-	none seen	18 X 10^6
Following heat shock	A.	50/<1	very little	granular	little	170 X 10^6
	Aa.***	--****	very little	granular	none seen	36 X 10^6
Following final centrifugation and washing	A	30:1	very little	cellular & granular	moderate	260 X 10^6
	Aa.***	--****	very little	cellular & granular	none seen	15 X 10^6

* Direct Microscopic Count
** Combined contents of a., b., and c.
*** Supernatant
**** No vegetative cells seen

Cages were changed every 2-3 days in order to avoid transmission of anthrax organisms in fecal matter of dead mice to live mice. All dead mice were autoclaved at 15 p.s.i., 121°C., for 35 minutes. The dead mice were placed in cages containing paper. Each cage was individually wrapped with brown wrapping paper prior to being autoclaved. All autocalved mice were disintegrated by heat, after leaving the autoclave. All equipment exposed to these organisms was autoclaved in a similar manner. Precautions regarding exterior clothing and rubber gloves were followed in a manner similar to those used in the chlorine test water determinations. Rubber boots were also used in this laboratory and were vigorously rinsed in a chlorine water foot bath containing 500 p.p.m. free chlorine at pH 6.0 and 25°C. Tincture of iodine was used in sterilizing the area of inoculation and a concentrated solution of free chlorine, 3,000 p.p.m., pH 5.0 at room temperature, was used as a general disinfectant for rinsing the tables, racks, window sill and floor during and at the end of the investigation. A concentrated chlorine aerosol was used to disinfect the walls and ceiling of the experimental animal laboratory.

Autopsies of dead mice were periodically made and the cause of death evaluated. Smears, agar streaks, and reinoculation into new mice followed each autopsy.

Results were recorded and tabulated in terms of I.P. $LD_{50's}$ using the following concentrations of spores per 0.2 ml. injection: 500, 100, 50, 10, and 1. Swab tests, similar to those used in sterility tests in the "Standard Methods for the Examination of Dairy Products"(42), were made daily to designate the presence or absence of anthrax spores or vegetative cells in the experimental laboratories.

Pathogenicity to mice was definitely demonstrated at dosages of 500 to 10 spores, during a period of 27 through 90 hours, respectively. Tables V and VI show the arithmetic and Reed-Muench methodology(43) which were used in determining the $LD_{50's}$ present in the mouse titrations. The two methods agreed in the final estimation of $LD_{50's}$ present.

Resistance of Spores to Free Available Chlorine

Procedure:

1. Measure 1190 ml. of previously buffered and standardized test water in chemically clean, ammonia-free (demand free), sterile 2 liter Erlenmeyer flasks and place in the constant temperature water bath until the temperature becomes stabilized at the desired test temperature range (4° or 22°C.). Prepare 2 flasks (a,b)* for testing the germicidal action of the chlorine concentration used. Also prepare one flask using buffered test water with only the test spores (to be used as an initial numbers control throughout the test period).
2. Add 5.0 ml. of the stock germicide (chlorine) to each test flask except the initial numbers control flask. The stock germicide should be diluted to a strength which results in the desired free available chlorine content when 5.0 ml. are added to 1195 ml. of the test water.

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*a. One flask for controlling the pH and chlorine content, without the addition of test spores, over the entire test period.

b. One flask containing the desired pH reaction, chlorine concentration, and the presence of test spores.

Table V

Analyses of Data used for Computation of Bacillus anthracis LD_{50}
(Based on 10-Day Observation)

Dilution	Spores Present*	Mouse Results Dead/Alive	% Mortality
1:2	500	12/12	100%
1:10	100	8/12	66.6%
1:20	50	5/12	41.6%
1:100	10	2/12	16.6%
1:1000	1	0/12	0.0%

* Based on Standard Plate Count using Liver Extract Tryptose Agar.

Table VI

Reed-Muench Method of Summation (calculated live and dead mice)
Actual Data from Table V Used in Computing LD_{50}

Dilution	Spores Present	Totals*		Totals**		% Mortality
		Alive	Dead	Alive	Dead	
1:2	500	0	12	0	27	100%
1:10	100	4	8	4	15	78.8%
1:20	50	7	5	11	7	38.9%
1:100	10	10	2	21	2	8.7%
1:1000	1	12	0	33	0	0.0%

* Actual Data

** Reed-Muench Derived Data

3. Add 5.0 ml. of the spore suspension to each designated experimental flask, and the desired controls as follows: rotate the flasks, stopping just before the suspension is added, creating sufficient residual motion of the water to prevent pooling of the suspension at the point of contact with the water. Avoid touching pipette to the neck or side of the test flask during the addition of suspension. (Spore suspension should contain 30×10^6 spores/ml. Final concentration in test water should be approximately 100,000 spores/ml.)

4. Transfer 5.0 ml. portions of the test water to neutralizer blanks in triplicate, at exactly the following intervals of exposure during the 48 hour exposure period: 0, 15, and 30 minutes, 1, 2, 4, 6, 12, 24, and 48 hours; mix well after the transfer. During the 2 hour storage experiments, sample in triplicate at intervals of exposure of 0, 15, and 30 minutes, 1 and 2 hours. In the case of the initial numbers control and other test controls, transfers should be made at the same sampling intervals.

5. Plate from the three neutralizer blanks into sterile petri dishes in triplicate, using proper dilutions, and use the specific agar for the test spores employed. (For necessary dilutions in order to receive countable plates, use phosphate buffered dilution water(42). Use an agar (1.5%) overlay on all plates.

6. Incubate plates at 37°C., for 24-72 hours, counting them at the end of 24, 48, and again at 72 hours. (Increased incubation was necessary due to the long lag phase in transition of spores to vegetative cells.)
7. Sterility Controls: (to be included in each experimental trial) All sterility controls are poured agar plate determinations.
   a. Neutralizer blanks - 1 ml. from each tube per plate.
   b. Test water used - 1 ml. per plate; plant triplicate plates.
   c. Sterile distilled water - 1 ml. per plate.
   d. Agar control on each agar bottle used.
   e. Culture survival control - immediately after each test flask has been sampled, add 5 ml. of the "culture suspension control" to a neutralizer blank, then place 1 ml. in 99 ml. of dilution water and plant dilutions to determine whether the original bacterial concentration of suspension has remained constant during the experimental trial.
8. After counting plates, confirm that the surviving organisms were spore-formers as originally used by transfer to tyrosine agar slants, and preparation of slides for positive confirmation. Usually it is only necessary to pick from representative colonies if many survivors are present. In all instances, sufficient colonies should be confirmed to eliminate the possibility of contaminants.
9. Determine the pH initially on the test water used and on each individual test flask immediately after each sampling interval.
10. Determine the free and total chlorine initially and after each sampling interval.

Safety Precautions:

1. Extreme care was taken when testing B. anthracis spores. Use of gauze masks, rubber gloves, and full length "surgeons" gowns minimized the danger of receiving external or internal infections.
2. Careful bacteriologically screened ventilation was practiced.
3. All equipment exposed was autoclaved as soon as possible. In the case of large or fixed objects, surfaces were rinsed with a strong chlorine solution, 2,000-4,000 p.p.m.

RESULTS

Two Hour Storage Period

Results showing the percent survival for both test spores at all sampling intervals, under varying conditions of pH and temperature can be seen in Tables VII and VIII. Calculated results showing time required for 99.99% destruction of the test spores can be seen in Table IX.

At all pH reactions and temperatures studied, B. globigii spores proved to be more resistant to the action of free available chlorine than B. anthracis spores, with the exception of pH 10.5, where the comparative resistance of both spores appeared to be about the same.

Forty-Eight Hour Storage Period

The results from this study are shown in Tables X through XII. Tables X and XI demonstrate the sporicidal efficiency of free available chlorine upon the rate of survival of 22°C., at all of the sampling intervals. The results at 4°C. can be seen in Table XII. In all of the experiments reported, free available chlorine values were not measurably different from total chlorine values.

Table VII

Average Survival of Spore Suspensions
Exposed to Free Available Chlorine at Several pH Levels
and Held at 22°C. (Observed 2 hrs.)

	Average percentage surviving after exposure (mins.)					Av. free available chlorine(ppm) after exposure (mins.)	
	0	15	30	60	120	0	120
pH 6.2							
B. globigii	100	100	100.0	94.0	52.0	0.48	0.45
" "	100	91	18.0	0.30	<0.01	1.93	1.86
B. anthracis	100	4	0.04	<0.01	0.00	2.2	2.2
pH 7.2							
B. globigii	100	92	88.0	74.0	5.0	1.15	1.0
" "	100	87	18.0	0.20	<0.01	2.6	2.5
B. anthracis	100	56	5.0	<0.01	0.00	2.4	2.0
pH 8.6							
B. globigii	100	92	83.0	34.0	0.3	16.0	15.6
" "	100	100	62.0	0.60	<0.01	25.0	24.0
B. anthracis	100	64	11.0	0.08	<0.01	24.4	24.0
pH 10.5							
B. globigii	100	100	100.0	100.0	50.0	50.0	50.0
" "	100	100	100.0	98.0	16.0	214.0	210.0
" "	100	100	100.0	47.0	5.0	289.0	287.0
" "	100	100	100.0	25.0	0.02	454.0	454.0
B. anthracis	100	100	100.0	26.0	0.02	445.0	439.0

Table VIII

Average Survival of Spore Suspensions
Exposed to Free Available Chlorine at Several pH Levels
and Held at 4°C. (Observed 2 Hrs.)

	Average percentage surviving after exposure (mins.)					Av. free available chlorine(ppm) after exposure (mins.)	
	0	15	30	60	120	0	120
pH 6.2							
B. globigii	100	100	100	80.0	0.06	4.6	4.6
" "	100	100	55.0	0.8	<0.01	6.4	6.0
B. anthracis	100	8	<0.01	<0.01	0.00	7.2	7.0
pH 7.2							
B. globigii	100	100	62.0	7.0	0.2	6.0	6.0
" "	100	100	35.0	0.4	<0.01	9.2	9.1
B. anthracis	100	29	0.2	<0.01	<0.01	9.3	9.3
pH 8.6							
B. globigii	100	100	77.0	9.0	0.10	66.0	64.0
" "	100	91	49.0	0.5	<0.01	86.0	84.0
B. anthracis	100	27	0.3	<0.01	0.00	92.0	92.0
pH 10.5							
B. globigii	100	100	100	100	52.0	410	400
B. anthracis	100	100	100	100	49.0	400	400

Table IX

Time Required (Hours of Chlorine Contact) to Kill Spore Suspensions

Temp.	pH	Cl_2 (ppm)	Number of spores* at 0 Hrs.	1 Hr.	2 Hrs.	% killed at 1 Hr.	2 Hrs.	Calculated time for 99.99% kill (Hrs.)
				Bacillus globigii				
4°C.	6.2	6.4-6.3	68,000	540	<1.0	99.20	>99.99	1.43
	7.2	9.2-9.1	100,000	350	2.0	99.66	>99.99	1.53
	8.6	85.6-84.4	110,000	560	<1.0	99.48	>99.99	1.50
	10.5	410-408	93,000	88,000	49,000	5.40	47.50	-**
22°C.	6.2	1.9-1.8	110,000	330	<1.0	99.70	>99.99	1.52
	7.2	2.6-2.5	110,000	350	<1.0	99.68	>99.99	1.37
	8.6	24.8-23.8	120,000	730	3.0	99.36	>99.99	1.50
	10.5	454	120,000	30,000	22.0	75.67	99.98	2.11
				Bacillus anthracis				
4°C.	6.2	7.20-7.1	92,000	0.66	0.0	>99.99	100.00	0.48
	7.2	9.30-9.2	88,000	3.70	<1.0	>99.99	>99.99	0.83
	8.6	92.30-91.3	130,000	5.00	0.0	>99.99	100.00	0.83
	10.5	402-400	130,000	110,000	64,000	15.40	50.80	-**
22°C.	6.2	2.2	110,000	1.00	0.0	>99.99	100.00	0.60
	7.2	2.4-2.3	110,000	1.00	0.0	>99.99	100.00	0.82
	8.6	24.4-23.4	110,000	150.00	<1.0	99.86	>99.99	1.28
	10.5	445-443	110,000	29,000	26.0	74.41	99.98	2.17

* Per ml.

** Not determined due to questionable extrapolation to durations greater than the two-hour limit observed experimentally.

Table X

Average Survival of Spore Suspensions When Exposed to Free Available Chlorine at Two pH Levels and Held at 22°C. for 48 Hrs.

	Average percentage surviving after exposure (hours)										Av. free available chlorine(ppm) after exposure (hours)	
	0	0.25	0.5	1	2	4	6	12	24	48	0	48
pH 6.2												
B. globigii	100	100	100	100	100	55.0	24.0	0.3	0.03	0.01	0.2	0.01
" "	100	100	100	100	100	100.0	29.0	0.3	<0.01	<0.01	0.24	0.11
" "	100	100	100	100	100	12.0	0.6	<0.01	<0.01	<0.01	0.35	0.10
" "	100	0									23.0	23.0
B. anthracis	100	100	100	72	58	1.0	0.5	0.00	0.00	0.00	0.2	0.12
pH 7.2												
B. globigii	100	100	100	100	98	91.0	29.0	0.26	0.00	0.00	0.34	0.15
" "	100	100	100	100	100	16.0	0.6	<0.01	0.00	0.00	0.44	0.25
" "	100	85	85	75	29	0.3	0.01	<0.01	<0.01	0.00	0.55	0.40
" "	100	0									19.5	19.5
B. anthracis	100	100	100	78	51	27.0	0.02	<0.01	<0.01	0.00	0.33	0.19
" "	100	100	100	85	58	1.0	0.02	0.00	0.00	0.00	0.45	0.24

Table XI

Average Survival of Spore Suspensions When Exposed to
Free Available Chlorine at Several pH Levels and Held at 22°C. for 48 Hrs.

	Average percentage surviving after exposure (hours)										Av. free available chlorine(ppm) after exposure (hours)	
	0	0.25	0.5	1	2	4	6	12	24	48	0	48
pH 8.6												
B. globigii	100	100	86	86	79	79.0	58.0	23.0	0.06	<0.01	1.2	0.9
" "	100	100	100	100	96	88.0	49.0	0.3	<0.01	0.00	2.3	1.8
" "	100	2	--*	--*	--*	0.0	0.0	0.0	0.0	0.00	19.7	19.7
B. anthracis	100	100	100	100	100	25.0	3.0	0.01	<0.01	<0.01	2.4	1.7
pH 9.6												
B. globigii	100	100	100	100	100	90.0	20.0	<0.01	<0.01	<0.01	15.6	14.6
pH 10.3												
B. globigii	100	100	100	100	100	100.0	100.0	92.0	58.0	0.10	5.5	4.3
" "	100	100	100	100	100	100.0	41.0	3.0	<0.01	0.00	23.0	22.0
" "	100	100	100	100	100	50.0	8.0	<0.01	<0.01	<0.01	25.0	23.0
B. anthracis	100	100	100	100	100	54.0	44.0	12.0	<0.01	0.00	20.0	18.4
pH 11.0												
B. globigii	100	85	--*	--*	--*	--*	9.0	1.0	0.02	<0.01	19.4	19.0

*No samples taken.

Table XII

Survival of Spore Suspensions When Exposed to
Free Available Chlorine at Several pH Levels and Held at 4°C. for 48 Hrs.

	Average percentage surviving after exposure (hours)											Av. free available chlorine(ppm)after exposure (hours)	
	0	0.25	0.5	1	2	4	6	12	24	48	56*	0	48
pH 6.2													
B. globigii	100	100	100	100	100	82	61.0	0.9	<0.01	<0.01		0.54	0.42
" "	100	100	100	100	100	96	40.0	0.3	<0.01	<0.01		0.64	0.58
" "	100	100	100	100	88	76	27.0	0.1	<0.01	<0.01		0.68	0.63
B. anthracis	100	100	100	83	L.A.	L.A.	0.2	<0.01	0.00	0.00		0.59	0.54
" "	100	100	100	81	25	2	<0.01	<0.01	0.00	0.00		0.65	0.60
pH 7.2													
B. globigii	100	100	100	100	100	100	62.0	2.0	<0.01	<0.01		0.56	0.47
" "	100	100	100	100	100	100	43.0	0.8	<0.01	<0.01		0.73	0.54
B. anthracis	100	100	100	85	L.A.	L.A.	2.0	<0.01	<0.01	<0.01		0.69	0.62
" "	100	100	100	79	79	16	4.0	<0.01	0.00	0.00		0.74	0.68
pH 8.6													
B. globigii	100	100	100	100	100	100	100.0	70.0	5.0	L.A.	0.01	3.7	3.2
" "	100	100	100	100	100	98	98.0	26.0	0.3	<0.01		5.6	5.5
B. anthracis	100	100	100	100	100	100	47.0	0.14	<0.01	0.00		4.8	4.7
pH 10.3													
B. globigii	100	100	100	100	100	100	81.0	46.0	42.0	1.0	0.6	28.0	26.0
" "	100	100	100	100	100	100	100.0	100.0	5.0	<0.01		46.0	45.0
B. anthracis	100	100	100	100	100	100	100.0	47.0	2.0	0.00		48.0	46.0

L.A. = Laboratory Accident
* = Extra Sampling Interval

The results as demonstrated in the above named tables indicate that B. globigii spores are more resistant than anthrax spores at lower pH ranges and have about the same resistance at pH 10.3.

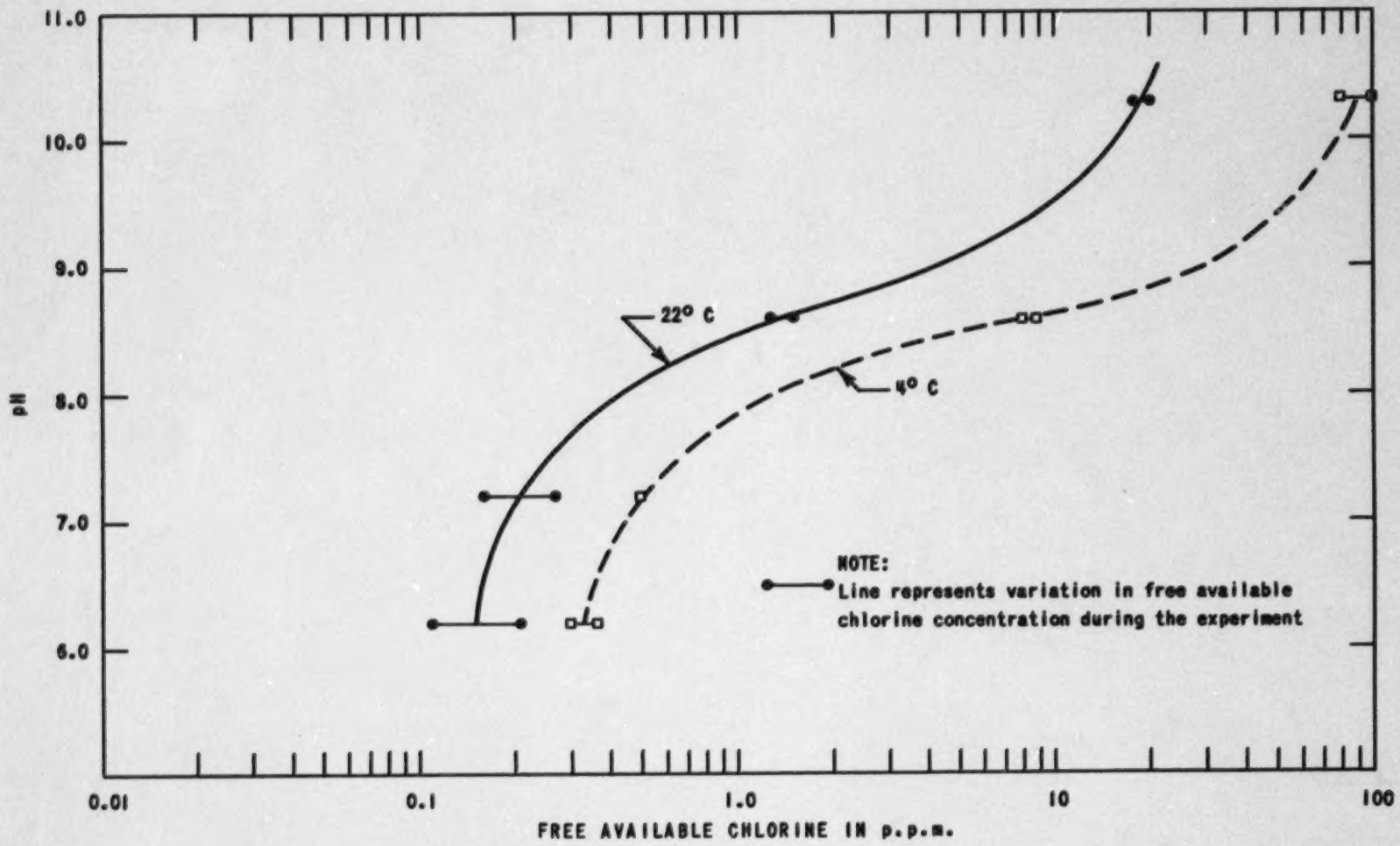
DISCUSSION OF RESULTS

Portions of the data presented in the tables have been shown graphically for purposes of discussion of the results obtained and of demonstrating the influence of certain variable factors on the resistance of the spores of B. globigii and B. anthracis, to free available chlorine. All of the data concerned with one variable could not be shown in any one chart without confusion because of the number of variables involved. As a result, though demonstrating the particular point in question, the data selected for presentation in the charts may not meet the full requirement of a particular situation.

Effect of Temperature

During the observarions conducted over a two hour holding period, it was found that in order to achieve a 99.99% kill of the test spores, it was necessary to increase the amount of free available chlorine by at least three-fold at 4°C., as compared to 22°C.

Figure 1 illustrates the influence of test temperature upon the concentration of free available chlorine required to obtain a 99.99% inactivation of B. globigii spores in 24 hours at all hydrogen-ion concentrations examined. At the higher pH levels, the temperature effect is greater than at the lower pH levels. At all hydrogen-ion concentrations studied below pH 9.5, B. anthracis spores were less resistant than B. globigii spores to the action of equivalent concentrations of free available chlorine at 22° and 4°C.



EFFECT OF TEMPERATURE UPON FREE AVAILABLE CHLORINE REQUIRED TO PRODUCE A 99.99% KILL OF B. GLOBIGII SPORES IN 24 HOURS AT VARYING pH

Figure I

The influence of temperature upon the sporicidal concentration of free chlorine for both test spores was consistent at all hydrogen-ion concentrations, and times.

Effect of pH

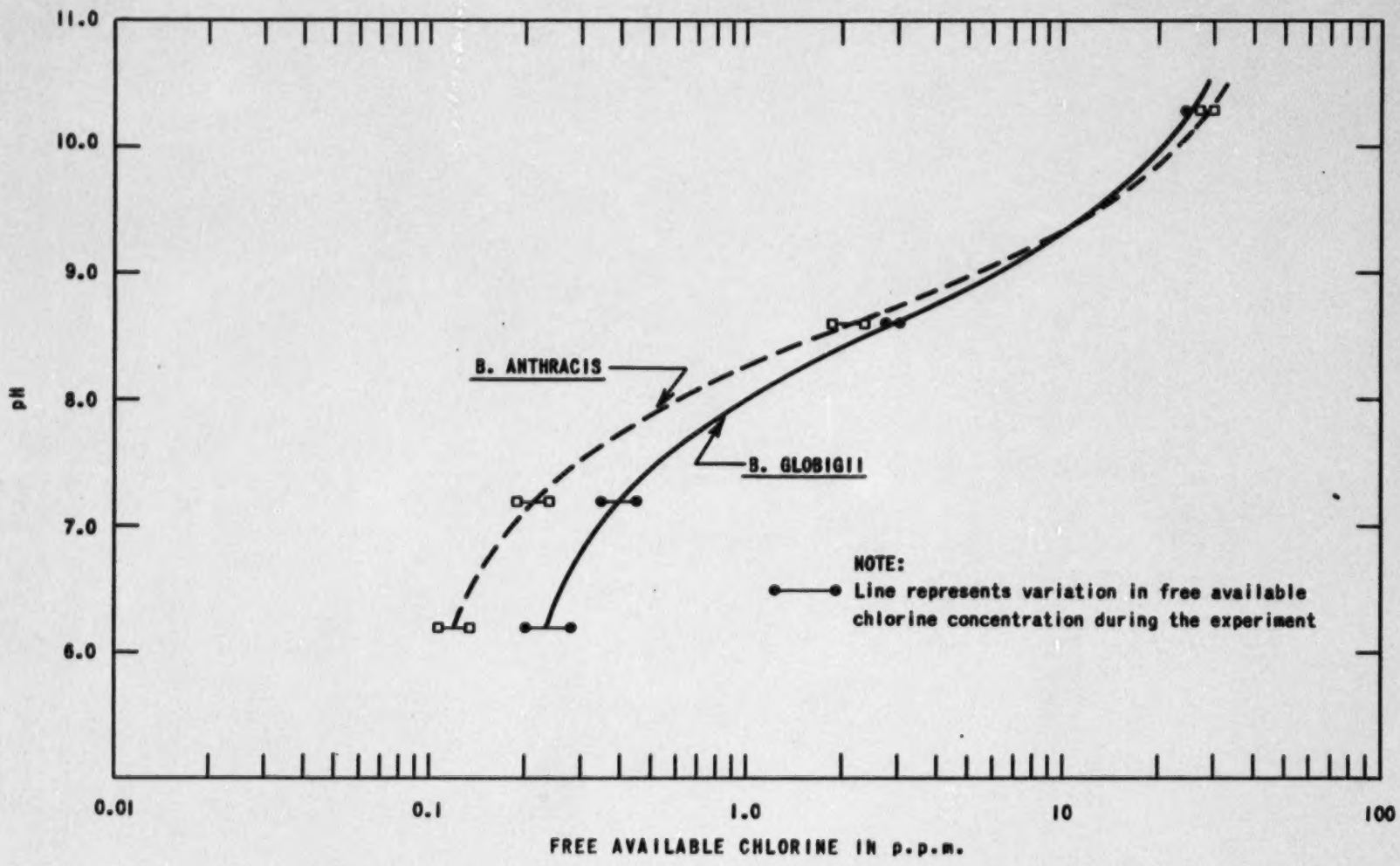
The marked difference in the concentration of free available chlorine required to effect a 99.99% inactivation at all pH levels was seen throughout all of the observations. As the pH level increased from 6.2 to 10.5, increased amounts of free available chlorine were required to kill the spores.

Data on the influence of varying hydrogen-ion concentrations are shown in Figure 2. At lower pH levels B. globigii spores were markedly more resistant than spores of B. anthracis. As the hydrogen-ion concentration decreased, the relative resistance of the two test spores approached unity. At pH 10.3, B. anthracis spores appeared to be slightly more resistant than B. globigii spores.

The influence of pH upon the destruction of B. globigii and B. anthracis spores can be seen most clearly in the results of the 48-hour exposure study since there were more sampling intervals, than in the 2-hour exposure observations.

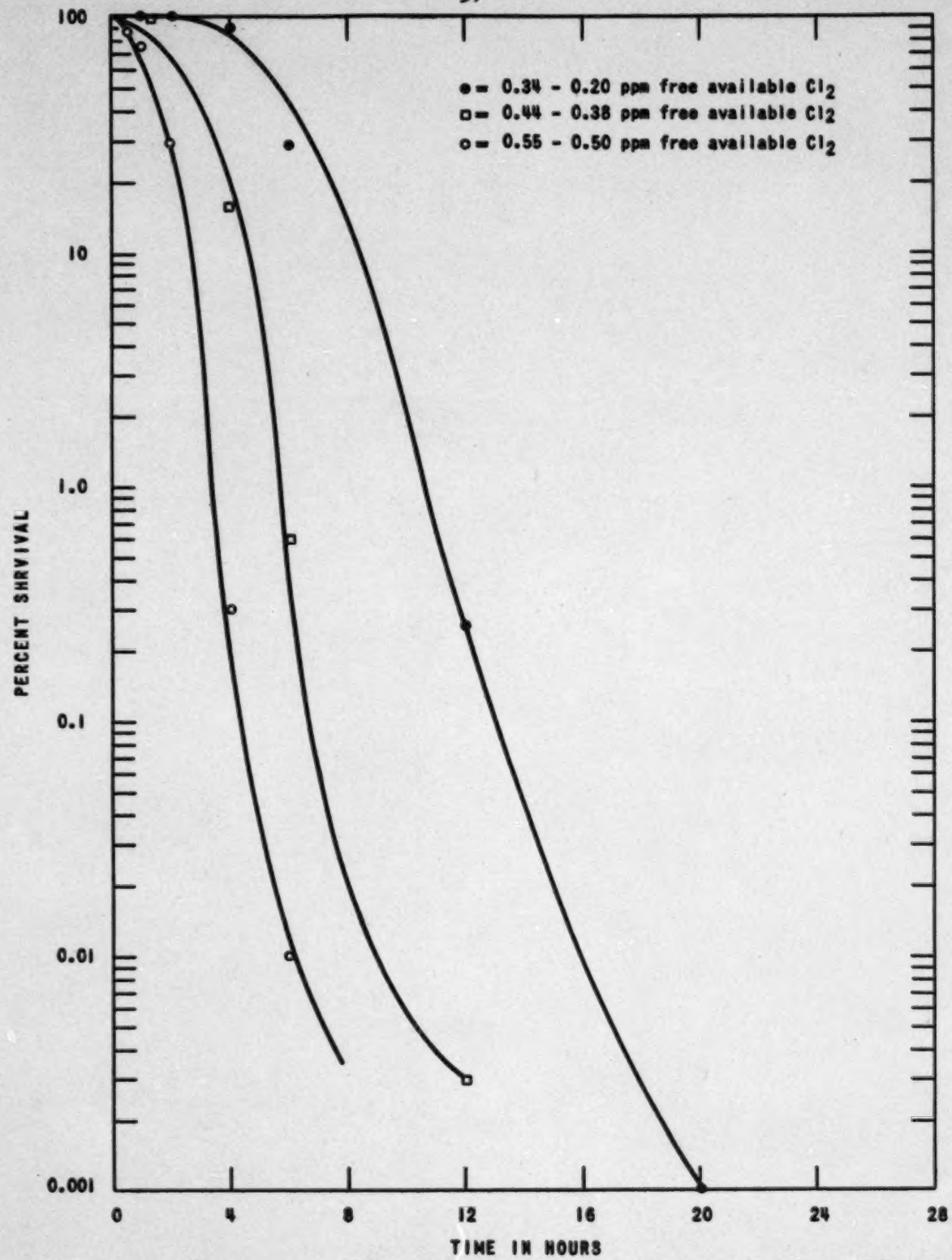
Effect of Time of Exposure

Figure 3 shows typical percent survival versus exposure time curves for B. globigii and B. anthracis. The time lag, before the effect of the chlorine on spores became apparent, was noticed at all temperatures and hydrogen-ion concentrations, but was less pronounced with the B. anthracis than with B. globigii. Figure 3 shows that at pH 7.2 and 22°C, 0.34, 0.44 and 0.55 p.p.m. free available chlorine required 3, 0.5 and 0.25 hours, respectively, before inactivation of B. globigii spores became evident.



COMPARISON OF FREE AVAILABLE CHLORINE REQUIRED FOR 99.99% KILL OF B. GLOBIGII AND B. ANTHRACIS SPORES IN 12 HOURS AT 22° C WITH VARYING pH

Figure 2



TYPICAL SURVIVAL CURVES FOR B. GLOBIGII SPORES AT pH 7.2 AND 22° C WITH VARYING FREE AVAILABLE CHLORINE CONCENTRATIONS

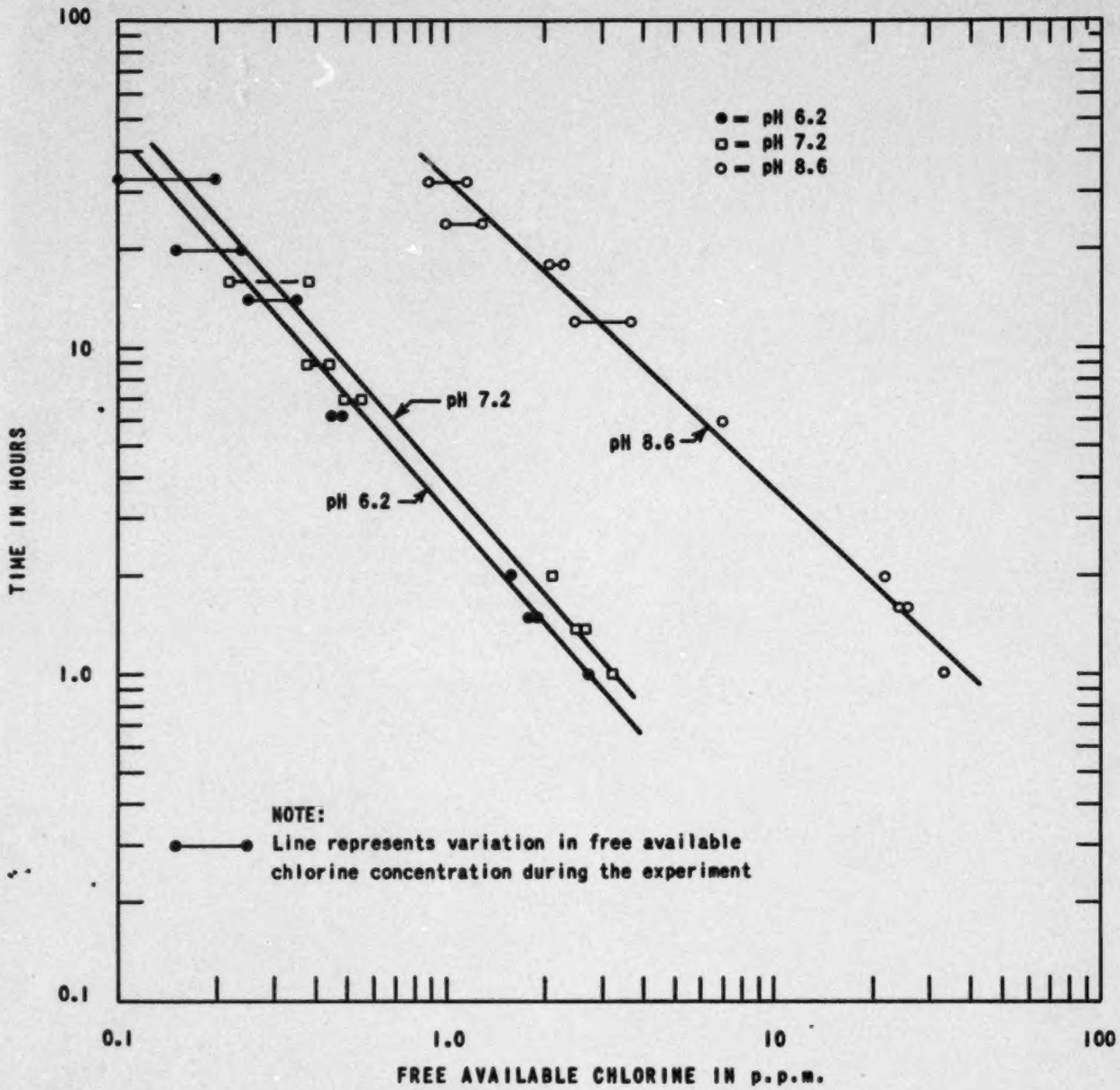
Figure 3

The practice of treating the disinfection process using first order kinetics (Chick's Law) is not justified in this case.

In all of these tests, there was some decrease in the initial free available chlorine concentrations during the exposure period. As a result, although some of the graphs may indicate that low concentrations of free available chlorine resulted in 99.99% destruction at the end of 24 or 48 hours, the higher initial free available chlorine concentrations were probably largely responsible for the resulting inactivation of both test spores used. If constant free available chlorine concentrations could be maintained, the times required for 99.99% destruction would be shorter than those observed under the conditions of these evaluations. However, in water plant practice the decrease in chlorine concentration with storage would be greater than was experienced in these tests.

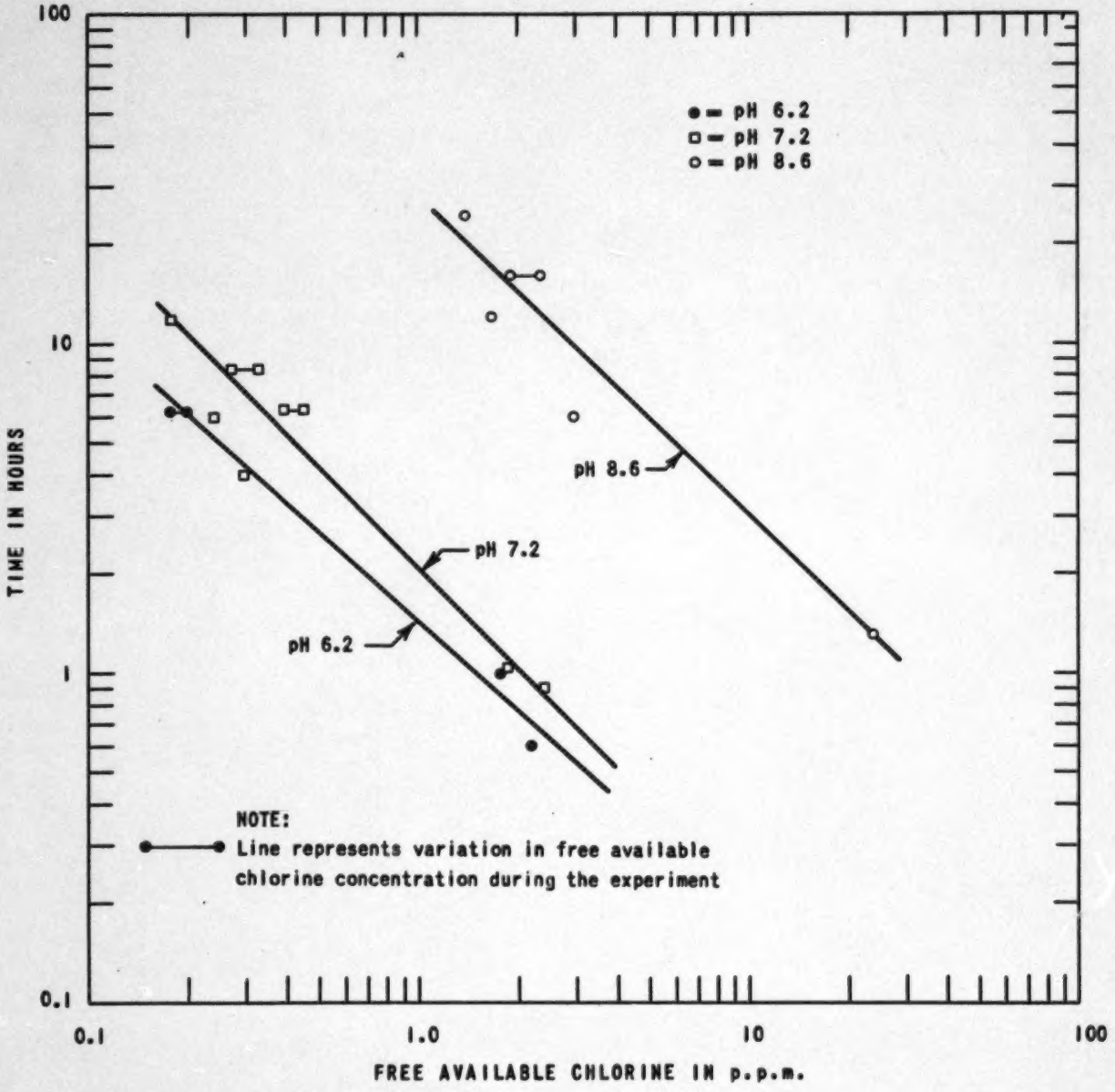
Figures 4-7 indicate the length of exposure which would be required to inactivate 99.99% of B. globigii and B. anthracis spores at 22° and 4°C., using varying amounts of free available chlorine. As plotted in Figure 4, at pH's 6.2, 7.2, and 8.6, 0.58, 0.7, and 6.0 p.p.m. free available chlorine, respectively, were required for inactivation of B. globigii spores within 6 hours at 22°C. In order to inactivate these spores in 1 hour, 2.7, 3.2, and 39 p.p.m. free available chlorine were required at pH levels of 6.2, 7.2, and 8.6, respectively.

Under parallel conditions of treatment, B. anthracis spores required 0.2, 0.36, and 4.9 p.p.m. free available chlorine at pH levels of 6.2, 7.2 and 8.6, respectively, for 99.99% inactivation in 6 hours as shown in Figure 5. Where inactivation was desired at the end of 1 hour exposure, the free available chlorine concentration required at pH levels 6.2, 7.2, and 8.6 was 1.5, 2.1, and 31 p.p.m., respectively.



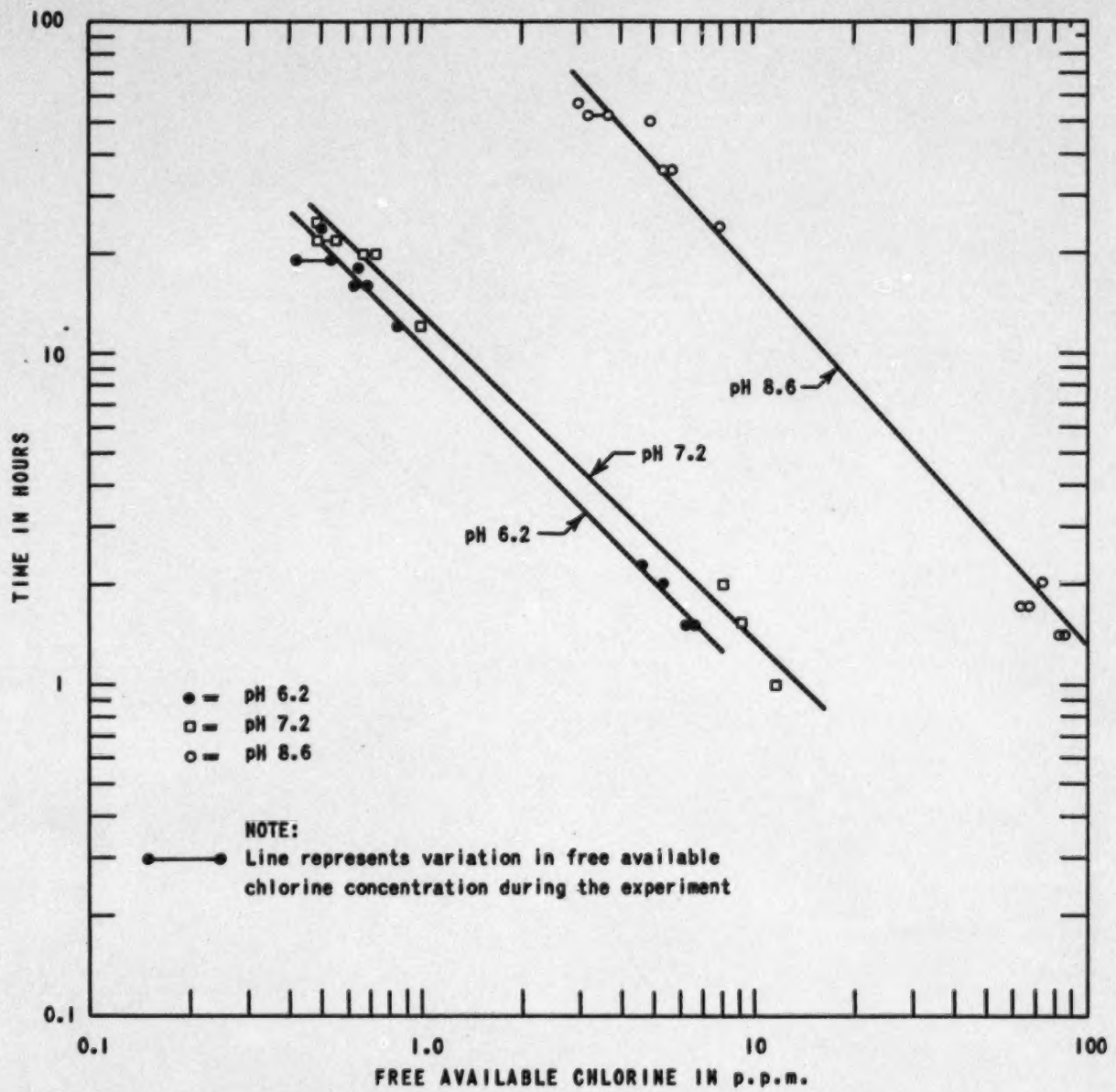
FREE AVAILABLE CHLORINE VS. TIME FOR 99.99% KILL
OF B. GLOBIGII SPORES AT VARYING pH AND 22° C

Figure 4



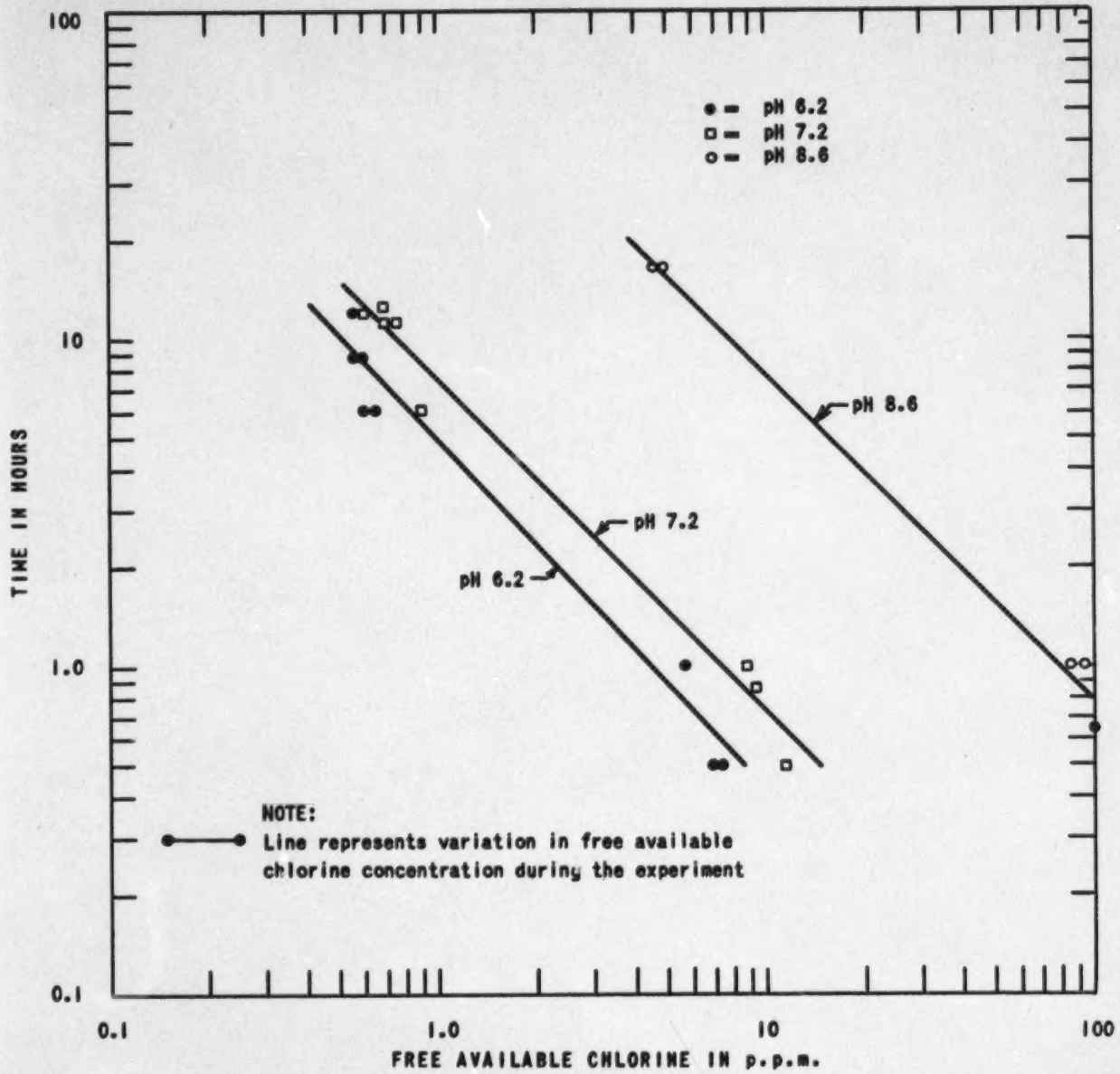
FREE AVAILABLE CHLORINE VS. TIME FOR 99.99% KILL OF B. ANTHRACIS SPORES AT VARYING pH AND 22° C

Figure 5



FREE AVAILABLE CHLORINE VS. TIME FOR 99.99% KILL
OF B. GLOBIGII SPORES AT VARYING pH AND 40° C

Figure 6



FREE AVAILABLE CHLORINE VS. TIME FOR 99.99% KILL OF B. ANTHRACIS SPORES AT VARYING pH AND 4° C

Figure 7

As previously noted, temperature greatly influenced the time required to achieve inactivation of the test spore suspensions. At 4°C., almost four times as much time was required to achieve 99.99% inactivation of B. globigii spores at all pH levels, as can be seen in Figure 6, using equivalent concentrations of free available chlorine that were present at 22°C. B. anthracis spores required three times as much exposure under similar conditions at 4°C., as at 22°C. (See Figure 7.)

In figures 4-7 inclusive the lines have slopes near to unity. Thus in the formula

$$C^n t = K$$

where C is the free available chlorine concentration required for 99.99% kill

t is the exposure time required

K is a constant for a given organism, pH and temperature

the exponent n is approximately one for both test organisms.

Effect of Hypochlorous Acid Concentration

The reaction between chlorine and water can be represented as follows:

$$Cl_2 + H_2O \longrightarrow HOCl + HCl$$

$$HOCl \rightleftharpoons H^+ + OCl^-$$

Hypochlorous acid has an ionization constant of about 3.5×10^{-8} at 22°C and 2.2×10^{-8} at 4°C.[31]. Thus at pH levels below neutrality the bulk of the hypochlorous acid is in unionized form and at higher pH levels it is largely ionized. The bactericidal effectiveness of HOCl is much greater than that of the OCl^- ion. Their relative effectiveness in killing spores is of the order of 100 to 1.

Figures 8 and 9 show concentrations of HOCl required for 99.99% kill of B. globigii and B. anthracis spores at 4° and 22°C. for various contact times. HOCl concentrations are computed from the observed free chlorine concentrations using the above ionization constants. The effect of pH is largely eliminated by this transformation and the data at pH 6.2, 7.2, and 8.6 can be well fit by a straight line. As in Figures 4-7, the lines are nearly parallel for both test organisms and both temperatures. The slope of the lines is approximately 1.1.

Thus the data are well represented by the equation

$C^n t = K$

where C is the concentration of HOCl in p.p.m. required for 99.99% kill

t is the time in hours

n is 1.1

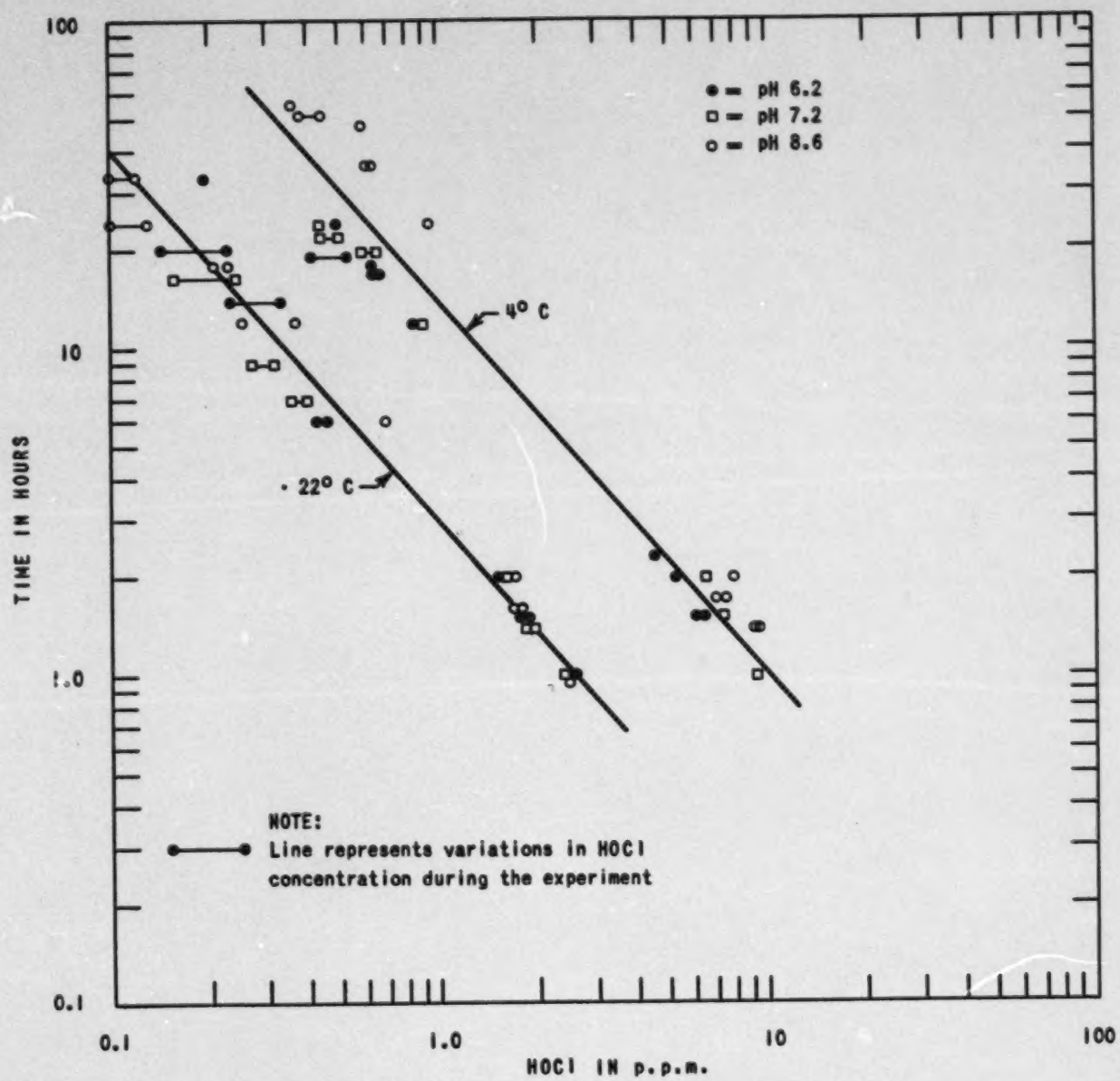
K is 3 for B. globigii at 22°C.

14 for B. globigii at 4°C.

1.4 for B. anthracis at 22°C.

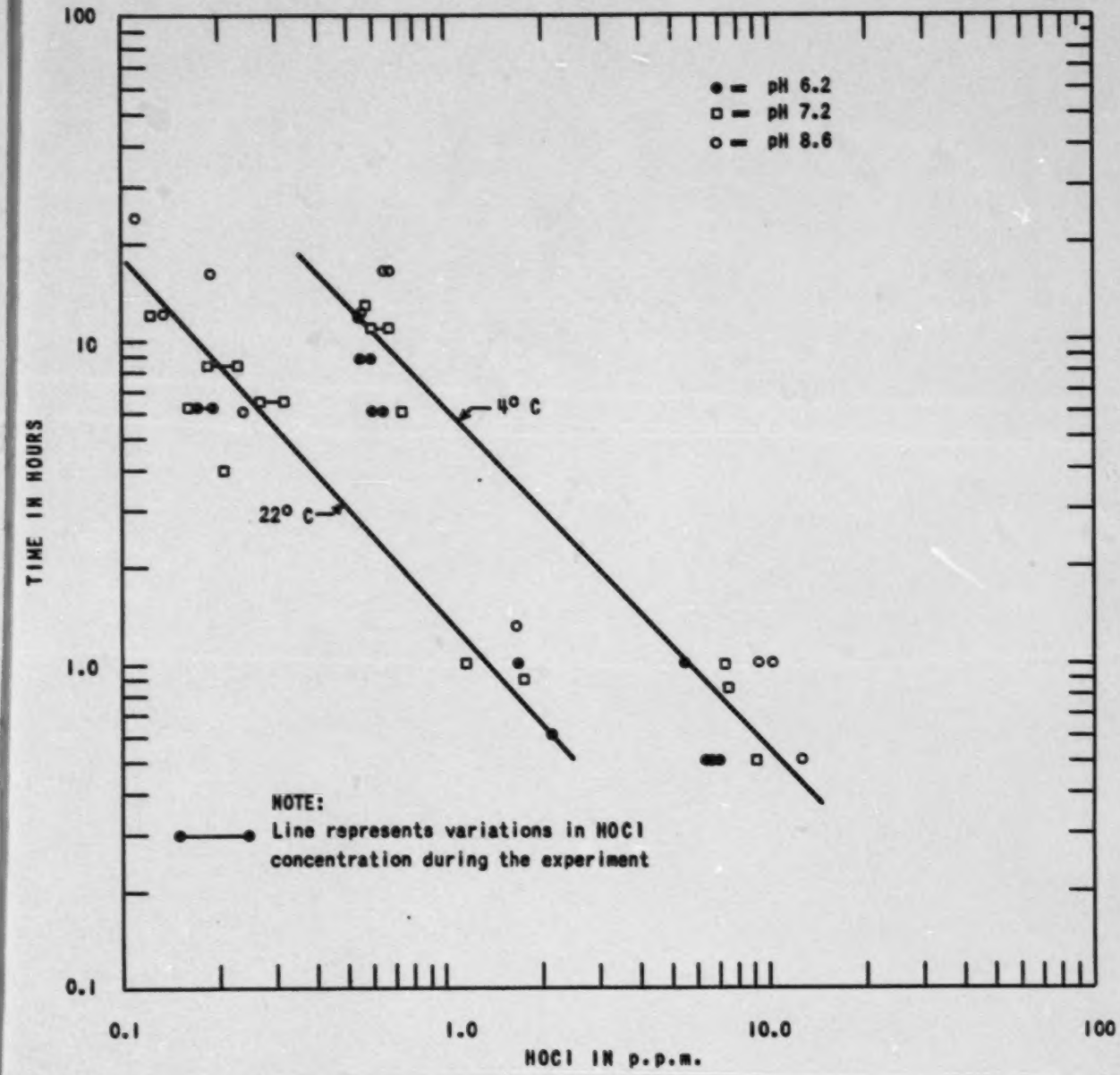
6.2 for B. anthracis at 4°C.

This formula would not apply beyond the range of the data on which it is based. It is particularly not applicable for times less than one hour. It would give conservatively high estimates of times and concentrations required at high pH levels since it neglects the effect of the OCl^- ion.



HOCl VS. TIME FOR 99.99% KILL OF
B. GLOBIGII SPORES AT 4° C AND 22° C

Figure 8



HOCl VS. TIME FOR 99.99% KILL OF
B. ANTHRACIS SPORES AT 4° C AND 22° C

Figure 9

CONCLUSIONS

The data in these studies show the time of exposure of B. globigii and B. anthracis spores to free available chlorine required to kill 99.99% of the spores initially present. The exposure time required is dependent upon the hydrogen-ion concentration, temperature, and amount of chlorine-demanding substances present. The action of free available chlorine on these spores cannot be well represented by a first order reaction. There is an initial lag period during which the spores are little affected by free available chlorine. The concentrations of free available chlorine required to kill spores are several orders of magnitude greater than those required to destroy vegetative cells under similar conditions of pH, temperature, and exposure time.

This study indicates that free available residual chlorination of water supplies would need to be carried on at pH levels of 7.0 or less in order effectively to destroy B. globigii spores within reasonable lengths of time and at practicable chlorine concentrations. Spores appear to be the most resistant bacteriological entity to the action of free available chlorine. As pH increases above neutrality, the concentration of free available chlorine required increases greatly. Adjustment of pH with acid or acid salts would be more economical than providing the necessary additional chlorine unless very long contact times are available.

Using B. globigii as a significant "index" organism, it can readily be seen from the results indicated in this report, that the amount of free available chlorine required to destroy these spores, under specified conditions of contact time, hydrogen-ion concentration, and temperature, will provide a safe margin of protection. Only as waters approach the high pH levels, in

excess of pH 8.6, as seen in those treatment plants employing lime-soda processes for softening water, do they reach a level or plateau where the resistance of B. globigii and B. anthracis spores to free chlorine is apparently equal. Adjustment of the hydrogen-ion concentration would provide a greater margin of safety.

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